J Acupunct Meridian Stud 2011;4(4):225–235

RESEARCH ARTICLE

Anti-invasive Activity of Ethanol Extracts of *Ganoderma lucidum* through Tightening of Tight Junctions and Inhibition of Matrix Metalloproteinase Activities in Human Gastric Carcinoma Cells

Kyung-Jun Jang [1], In-seok Son [1], Dong Yeok Shin [2], Hyun-Min Yoon [1,*], Yung Hyun Choi [2,3,*]

[1] *Departments of Acupuncture & Moxibustion, Dongeui University College of Oriental Medicine, Busan, South Korea*
[2] *Department of Biochemistry, Dongeui University College of Oriental Medicine, Busan 614-052, South Korea*
[3] *Department of Biomaterial Control (BK21 program), Dongeui University Graduate School and Blue-Bio Industry Regional Innovation Center, Dongeui University, Busan 614-714, South Korea*

Available online Oct 20, 2011

Received: Mar 16, 2011
Revised: May 2, 2011
Accepted: Aug 29, 2011

KEYWORDS
AGS;
Ganoderma lucidum;
invasion;
tight junction

Abstract
This study investigated the effect of ethanol extracts of *Ganoderma lucidum* (EGL) on the correlation between tightening of the tight junctions (TJs) and the anti-invasive activity in human gastric adenocarcinoma AGS cells to elucidate further the possible anticancer mechanisms that *G lucidum* exerts. Within the concentrations of EGL that were not cytotoxic, EGL markedly inhibited the cell motility and invasiveness in a concentration-dependent manner. The activities of matrix metalloproteinases (MMP)-2 and MMP-9 in AGS cells were dose-dependently inhibited by treatment with EGL, and this was correlated with a decrease in expression of their mRNA and proteins and the upregulation of the expression of the tissue inhibitors of metalloproteinases. The anti-invasive activity of EGL was also found to be associated with the increased tightness of the TJ, which was demonstrated by an increase in transepithelial electrical resistance. Additionally, EGL repressed the levels of the claudin family members, which are major components of TJs that play a key role in the control and selectivity of paracellular transport. Furthermore, the levels of E-cadherin, a type I transmembrane glycoprotein, were inhibited by EGL treatment, however, those of snail, an epithelial to mesenchymal transition regulator and zinc finger transcription factor, were concentration-dependently increased in response to EGL treatment. Although further studies are needed, the present study indicates that TJs and MMPs are crucial targets of EGL-induced anti-invasiveness in human gastric cancer AGS cells.

* Corresponding authors. Department of Acupuncture & Moxibustion, Dongeui University College of Oriental Medicine, Busan 614-052, South Korea.
E-mail: yhmin@deu.ac.kr (H.-M. Yoon), choiyh@deu.ac.kr (Y.H. Choi)

doi:10.1016/j.jams.2011.09.013

1. Introduction

Metastasis is a sequential multistep process that ultimately leads to the outgrowth of the cancer from one organ to another. This process involves several steps: invasion of adjacent tissues, intravasation, transport of cancer cells through the circulatory system, arrest at a secondary site, extravasation, and growth in a secondary organ [1—3]. Therefore, the inhibition of tumor cell migration and invasion are important mechanisms in the antimetastatic properties of anticancer drugs.

Recently, the many clinical and laboratory data indicated that the inverted expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) suggest that they function as key regulators in cancer invasion, metastasis, and progression. MMPs are a family of zinc-dependent endopeptidases. They are known to process a broad spectrum of cell surface molecules and to function in several important biological processes. MMPs are collectively capable of cleaving virtually all extracellular matrix (ECM) substrates, and the degradation of the matrix is a key event in the invasion and the metastasis of potentially malignant lesions [4,5]. Among the various MMPs, MMP-2 and MMP-9 (known as gelatinase A and gelatinase B, respectively) seem to play an important role in tumor invasion and metastasis and are highly expressed in epithelial cancer cells, including gastric carcinoma cells [6,7]. TIMPs are naturally occurring inhibitors of MMPs. They inhibit the catalytic activity of MMPs by binding to activated MMPs and controlling the breakdown of the ECM [8]. TIMPs can also inhibit proliferation, invasion, and metastasis of malignant cells. Because disturbances in the balance between MMPs and TIMPs can be found in various pathologic conditions, including cancer [9], the balance between MMPs and TIMPs plays a vital role in maintaining the integrity of healthy tissues; MMP inhibitors, as well as TIMP activators, are expected to be useful chemotherapeutic agents for the treatment of malignant cancer.

In addition, cell adhesion between neighboring epithelial cells is a crucial and tightly controlled process. In epithelial cells, several specialized and distinct intercellular structures, including the gap junction, tight junction (TJ), adherens junction (AJ), and desmosome, are responsible for the establishment of contact between neighboring cells. Among them, the most apical of these are the TJs, which form tight seals between cells and the intercellular space. Within a cell, TJs act as a fence that blocks the movement of membrane proteins between the apical and basolateral surfaces thereby maintaining the apical—basal polarity of the cell [10,11]. TJ strands also function as a gate that regulates paracellular movement of ions and small molecules between the apical and basolateral surfaces of the layer. In precancerous lesions of the epithelia and cancerous epithelia, TJ strands become disorganized or lost altogether and TJs become "leaky," as indicated by decreased resistance to electrical current, otherwise known as transepithelial electrical resistance (TER), and increased paracellular permeability of the markers [10,11]. Claudins, which are key integral membrane proteins that form the backbone of TJs, can form homodimers or heterodimers to produce paired strands between adjacent cells. The paired strands act as a barrier to the paracellular flux of water and solutions and the transmigration of other cells thereby determining the characteristic permeability properties of the different epithelial tissues [12,13]. Recent studies have provided evidence that claudins are aberrantly expressed in various cancers and are associated with the development and progression of cancer, which suggests that they have key cellular functions that are distinct from their roles in TJ-complexes. However, the exact role of claudin over-expression and the functional importance of these proteins in the development of cancers remain unclear.

Many studies have indicated that mushrooms are proving to be novel and rich sources of bioactive compounds. Among them, *Ganoderma lucidum* (靈芝, *lingzhi* in Chinese or *reishi* in Japanese) is a polypore mushroom that grows on the lower trunks of deciduous trees. This mushroom has been widely used in traditional Oriental medicine in tonics for promoting longevity and health for thousands of years in Asian countries, including China, Japan, and Korea [14—17]. The pharmacological activities of *G lucidum*, especially its intrinsic immunomodulating, anti-inflammatory, and anti-tumor properties, have been extensively documented [17—20]. In particular, several studies have demonstrated that various extracts of *G lucidum* act as anticancer agents by interfering with the cell cycle progression, inducing apoptosis, and suppressing angiogenesis in human cancer cells [21—28]. However, the precise biochemical mechanisms underlying *G lucidum* extracts-induced anti-invasiveness and antimetastasis have not yet been clarified.

Therefore, the present study attempted to elucidate the antimetastatic potentials of the ethanol extracts of *G lucidum* (EGL) in human gastric carcinoma AGS cells and the underlying intracellular signal transduction pathways involved in inhibition metastasis. The results of this study demonstrated that EGL inhibits the cell motility and invasion of AGS cells through the modulation of the activities of MMPs and the levels of TJ-associated factors.

2. Materials and methods

2.1. Cell culture

Human gastric carcinoma AGS cells were purchased from the American Type Culture Collection (Rockville, MD, USA). They were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and 1% penicillin—streptomycin in a 37 °C incubator with 5% CO_2.

2.2. Preparation of EGLs

EGLs were obtained from Dongeui University Oriental Hospital, Busan, Korea. The freeze-dried and milled fruiting bodies of *G lucidum* (200 g) were extracted with 25% ethanol (4 L) at room temperature for 10 hours using a blender. The extracts were filtered through a Whatman (Maidstone, UK) #2 filter, concentrated to 500 mL under vacuum, and then kept at −20 °C [28]. The EGL solution obtained was directly diluted in the medium before assay.

2.3. MTT assay

For the cell viability study, AGS cells were grown to 70% confluence. Then the cells were treated with various concentrations of EGL for 48 hours. Following treatment, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes.

2.4. Migration assay

For the migration assay, AGS cells were grown to confluence on 30-mm cell culture dishes coated with rat tail collagen (20 μg/mL, BD Biosciences, Bedford, MA, USA). A scratch was made in the cell layer with a pipette tip. After washing with phosphate buffered saline (PBS), serum-free media (to prevent cell proliferation) containing 0.5% or 1.0% of EGL was added. Photographs of the wounded area were taken immediately after the scratch was made and again 48 hours later to monitor cell movement into the wounded area [29].

2.5. *In vitro* invasiveness assay

Matrigel invasion assays were used to assess the ability of AGS cells to penetrate ECM in the presence or absence of EGL. Briefly, cells were exposed to EGL for 6 hours, and treated cells (50,000) were then plated onto the apical side of the Matrigel-coated filters in serum-free medium containing 0.5% or 1.0% of EGL. Medium containing 20% fetal bovine serum was placed in the basolateral chamber to function as a chemoattractant. After 48 hours, cells on the apical side were wiped off with a cotton-tipped swab. Cells on the bottom of the filter were stained with hematoxylin and Eosin Y (Sigma) and counted (three fields of each triplicate filter) using an inverted microscope.

2.6. Measurement of TER

TER was measured with an EVOM Epithelial Tissue Voltohmmeter (World Precision Instruments, FL, USA) equipped with a pair of STX-2 chopstick electrodes (World Precision Instruments). Briefly, AGS cells were seeded into the 8.0 μm pore-size insert (upper chamber) of a Transwell® (Corning Costar Corp., NY, USA) and allowed to reach full confluence after which fresh medium was replaced for further experiments. Inserts without cells, inserts with cells in medium, and inserts with cells with 0.5% or 1.0% of EGL were treated for 48 hours. Electrodes were placed at the upper and lower chambers, and the resistance was measured with the voltohmmeter.

2.7. RNA extraction and reverse transcription polymerase chain reaction

Total RNA was prepared using an RNeasy kit (Qiagen, La Jolla, CA, USA) and primed with random hexamers for the synthesis of complementary DNA with AMV reverse transcriptase (Amersham Co., Arlington Heights, IL, USA), according to the manufacturer's instructions with DNase I (1 U/μg RNA) pretreated total mRNA. Polymerase chain reaction (PCR) was carried out in a Mastercycler (Eppendorf, Hamburg, Germany) with the primers indicated in Table 1. Conditions for the PCR reactions were 1 × (94 °C for 3 min), 35 × (94 °C for 45 sec; 58 °C for 45 sec; and 72 °C for 1 min), and 1 × (72 °C for 10 min). Amplification products obtained by PCR were electrophoretically separated on a 1% agarose gel and visualized by ethidium bromide staining.

2.8. Protein extraction and western blot analysis

Total cell lysates from EGL-treated cells were prepared in an extraction buffer: 25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM ethylendiaminetetra acetic acid, 1% nonidet P-40, 0.1 mM sodium orthovanadate, 2 μg/mL leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Laboratories, Hercules, CA, USA). For western blot analysis, proteins (50 μg) were separated by 8∼13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 5% skim milk for 1 hour and then subjected to immunoblot analysis with the desired antibodies. The proteins were then visualized by the enhanced chemiluminescence method, according to the recommended procedure (Amersham Co.). Primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Calbiochem (Cambridge, MA, USA). Peroxidase-labeled donkey antirabbit immunoglobulin and peroxidase-labeled sheep antimouse immunoglobulin were purchased from Amersham Co.

2.9. Gelatin zymographic analysis of secreted MMPs

Following incubation with various concentrations of EGL for 48 hours, cell culture supernatants were collected and centrifuged at 400 × g for 5 minutes. Cell-free supernatant was mixed with 2X sample buffer (Invitrogen, CA, USA) and zymography was performed using precast gels (10.0% polyacrylamide and 0.1% gelatin). Following electrophoresis, gels were washed twice at room temperature for 30 minutes in 2.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA), subsequently washed in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 1 μm $ZnCl_2$, and 0.02% NaN_3 at pH 7.5, and then incubated in this buffer at 37 °C for 24 hours. Thereafter, the gels were stained with 0.5% (w/v) Coomassie brilliant blue G-250 (Bio-Rad) for 1 hour and then lightly destained in methanol:acetic acid:water (3:1:6). Clear bands appear on the Coomassie stained blue background in areas of gelatinolytic activity. Gels were scanned and images were processed by extracting the blue channel signal, converting it to black and white, and then inverting it for quantification of gelatinolytic activities from the integrated optical density [30].

Table 1 Sequences of the primer pairs employed in the RT-PCR.

Name		Sequence of primers
TIMP-1	sense	5′-TGG-GGA-CAC-CAG-AAG-TCA-AC-3′
	antisence	5′-TTT-TCA-GAG-CCT-TGG-AGG-AG-3′
TIMP-2	sense	5′-GTC-AGT-GAG-AAG-GAA-GTG-GAC-TCT-3′
	antisence	5′ATG-TTC-TTC-TCT-GTG-ACC-CAG-TC-3′
MMP-1	sense	5′-GAT-GTT-CAG-CTA-GCT-CAG-GAT-3′
	antisence	5′-AAG-GGA-TTT-GTG-CGC-ATG-TAG-3′
MMP-2	sense	5′-CTT-CTT-CAA-GGA-CCG-GTT-CAT-3′
	antisence	5′-GCT-GGC-TGA-GTA-GAT-CCA-GTA-3′
MMP-7	sense	5′-GGC-CCT-GTC-ACT-CCT-GAG-AT-3′
	antisence	5′-GGC-ATC-CAG-GTT-ATC-GGG-GA-3′
MMP-9	sense	5′-CGG-AGC-ACG-GAG-ACG-GGT-AT-3′
	antisence	5′-TGA-AGG-GGA-AGA-CGC-ACA-GC-3′
claudin-1	sense	5′-TCA-GCA-CTG-CCC-TGC-CCC-AGT-3′
	antisence	5′-TGG-TGT-TGG-GTA- AGA-GGT-TGT-3′
claudin-2	sense	5′-ACA-CAC-AGC-ACA-GGC-ATC-AC-3′
	antisence	5′-TCT-CCA-ATC-TCA-AAT-TTC-ATG-C-3′
claudin-4	sense	5′-TGG-ATG-AAC-TGC-GTG-GTG-CAG-3′
	antisence	5′-GAG-GCG-GCC-CAG-CCG-ACG-TA-3′
E-cadherin	sense	5′-GAA-CAG-CAC-GTA-CAC-AGC-CCT-3′
	antisence	5′-GCA-GAA-GTG-TCC-CTG-TTC-CAG-3′
snail	sense	5′-TAT-GCT-GCC-TTC-CCA-GGC-TTG-3′
	antisence	5′-ATG-TGC-ATC-TTG-AGG-GCA-CCC-3′
GAPDH	sence	5′-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3′
	antisence	5′-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3′

GAPDH = glyceraldehyde-3-phosphate; MMP = matrix metalloproteinase; TIMP = tissue inhibitors of metalloproteinase.

2.10. Statistical analysis

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student *t* test. A value of $p < 0.05$ was accepted as an indication of statistical significance. All of the figures shown in this article were obtained from at least three independent experiments.

3. Results

3.1. Inhibition of cell viability by EGL treatment in AGS cells

To determine if EGL decreases cell growth, AGS cells were incubated with various concentrations of EGL and the cell viability was then measured by an MTT assay. As shown in Fig. 1, after 48 hours of EGL treatment, low concentrations of EGL (0.25-1.00%) did not reduce cell proliferation, whereas a high concentration of EGL (1.5%) significantly caused a decrease in cell viability. When compared with the control, treatments with 1.0% and 1.5% of EGL caused approximately 6.5% and 21.5%, respectively, inhibition of cell growth.

3.2. Inhibition of cell migration by EGL in AGS cells

Low concentrations of EGL (less than 1.0%) did not affect cell viability and did not induce apoptotic cell death (data not shown), whereas high concentrations were cytotoxic. Therefore, we applied 0.5% and 1.0% of EGL for the optimal treatment concentrations to investigate whether EGL decreases the activity of cell migration and the invasion of AGS cells. Results of the migration assay demonstrated that treatment with 0.5% and 1.0% of EGL time-dependently delayed cell motility when compared with the controls (Fig. 2).

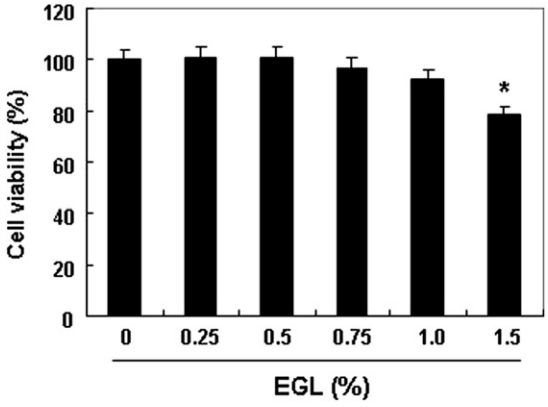

Figure 1 Effects of ethanol extracts of *G lucidum* (EGL) on the cell viability in AGS human gastric carcinoma cells. The cells were seeded at an initial density of 2.5×10^5 cells per 60-mm plate, incubated for 24 hours, and then treated with various concentrations of EGL for 48 hours. The cell viability was measured using an MTT assay. Each point represents the mean ± SD of three independent experiments. Significance was determined using a Student *t* test. * $p < 0.05$ versus untreated control.

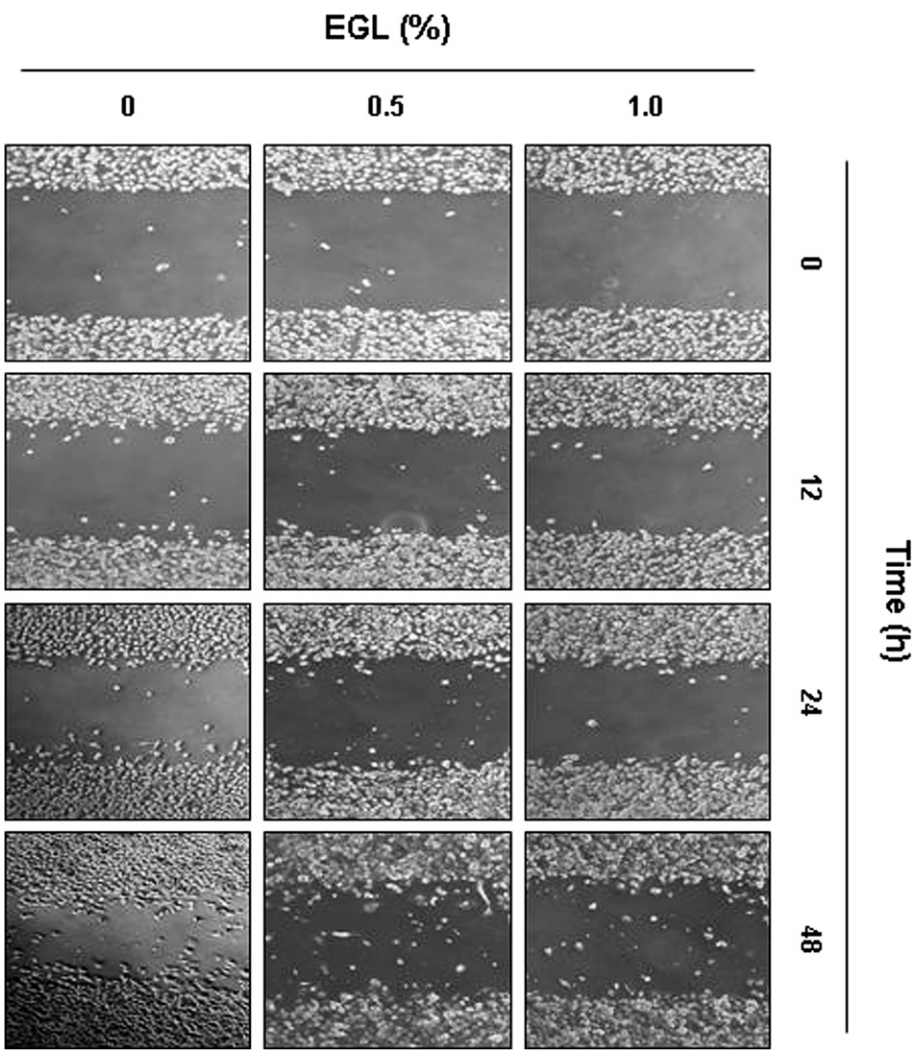

Figure 2 Inhibition of cell motility by ethanol extracts of *G lucidum* (EGL) in AGS cells. Cells were grown to confluency on 30-mm cell culture dishes and then a scratch was made through the cell layer with a pipette tip. After washing with phosphate buffered saline serum-free media (to prevent cell proliferation) containing either vehicle or EGL (0.5% or 1.0%) was added for the indicated tines. Photographs of the wounded area were taken for evaluation of cell movement into the wounded area.

3.3. Inhibition of cell invasion by EGL in AGS cells

Using a Boyden chamber invasion assay, we next examined if EGL decreases the activity of cell invasion. As shown in Fig. 3, EGL treatment markedly reduced cell invasion through the Matrigel chamber in a concentration-dependent manner. This suggests that the inhibitory effects of cell migration are associated with the inhibition of invasive activity in AGS cells.

3.4. Induction of TIMP-1 and TIMP-2 expression by EGL in AGS cells

Because migration influences metastasis and the invasion of the basement membrane is primarily mediated by the gelatinase MMPs and their inhibitors (TIMPs), we tested the effects of EGL on the levels of TIMP-1 and TIMP-2. The results of reverse transcription PCR (RT-PCR) showed that EGL concentration-dependently increased TIMP-1, as well as TIMP-2 mRNA levels. This was connected with a concurrent upregulation of their protein levels (Fig. 4), suggesting that the increased protein expression of TIMPs by EGL could inhibit the activity of MMPs.

3.5. Inhibition of the expression and activity of MMP-2 and MMP-9 by EGL in AGS cells

We next investigated the effects of EGL treatment on the levels of MMPs and their activities. The results of RT-PCR showed that EGL decreased MMP-2 and MMP-9 mRNA levels in a concentration-dependent manner without the alteration of other MMPs, such as MMP-1 and MMP-7 (Fig. 5A). In addition, we performed the gelatin zymographic analysis to examine whether the inhibitory effects on the mRNA levels of MMP-2 and MMP-9 were associated with the down-regulation of their activities. As indicated in Fig. 5C, EGL concentration-dependently decreased MMP-2 and MMP-9 activities, which was also connected with a concurrent

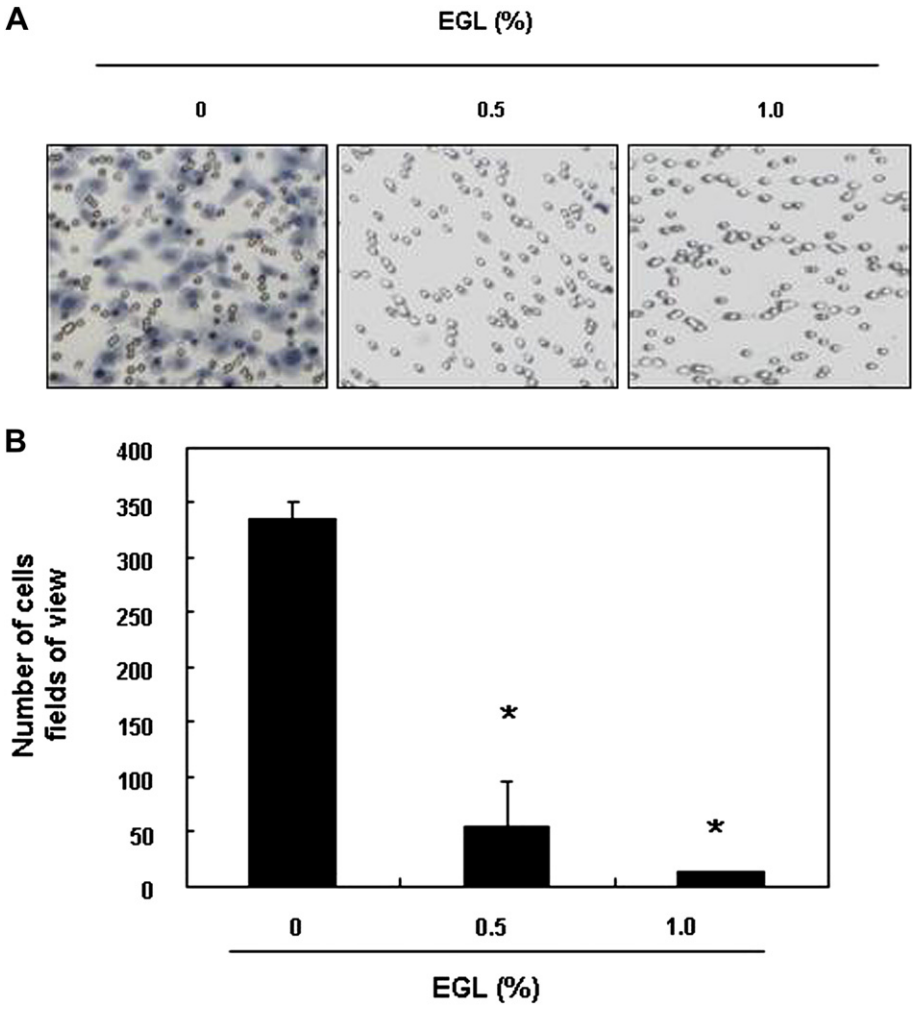

Figure 3 Effects of ethanol extracts of *G lucidum* (EGL) on cell invasion in AGS cells. (A) Cells pretreated with the indicated concentrations of EGL for 6 hours were plated onto the apical side of Matrigel-coated filters in a serum-free medium containing either vehicle or EGL. Medium containing 20% FBS was placed in the basolateral chamber to act as a chemoattractant. After 48 hours, the cells on the apical side were wiped off using a cotton-tipped swab Next, the cells on the bottom of the filter were stained using hematoxylin and Eosin Y and then counted. (B) Data are shown as the mean of triplicate samples and represent the invasive cell numbers compared with those of control cells. * $p < 0.05$ versus untreated control.

downregulation of their protein levels (Fig. 5B). These results suggest that the anti-invasive effect of EGL is associated with increased TIMP-1 and TIMP-2 levels, as well as the inhibition of MMP-2 and MMP-9 levels, and their activity in AGS cells.

3.6. Enhancement of the tightening of TJs by EGL in AGS cells

To examine the relationship between the tightening of TJS and the invasive activity of AGS cells treated with EGL, TER (a measure of TJ formation) values were measured using an EVOM Epithelial Tissue Voltohmmeter (World Precision Instruments). As shown in Fig. 6, the incubation of cells with EGL substantially increased their TER values in a concentration-dependent manner, suggesting that EGL increased TJs function in AGS cells.

3.7. Modulation of TJs-related factors by EGL in AGS cells

To elucidate the mechanism by which EGL enhances TJs activity and reduces invasive activity in AGS cells, we determined the levels of TJs and AJs components, as well as TJ regulators, such as claudins, E-cadherin, and snail, using RT-PCR and western blot analyses. As shown in Fig. 7, both the transcriptional and translational levels of claudins (claudin-1, claudin-2, and claudin-4), the most important components of the TJ [31], were markedly downregulated in EGL-treated cells in a time-dependent manner, suggesting that this modulation contributed to the tightening of TJs. In addition, the levels of E-cadherin, a type I transmembrane glycoprotein that regulates TJ and AJ formation [32,33], were markedly inhibited by EGL treatment. However, those of snail proteins, an epithelial to mesenchymal transition regulator and zinc finger

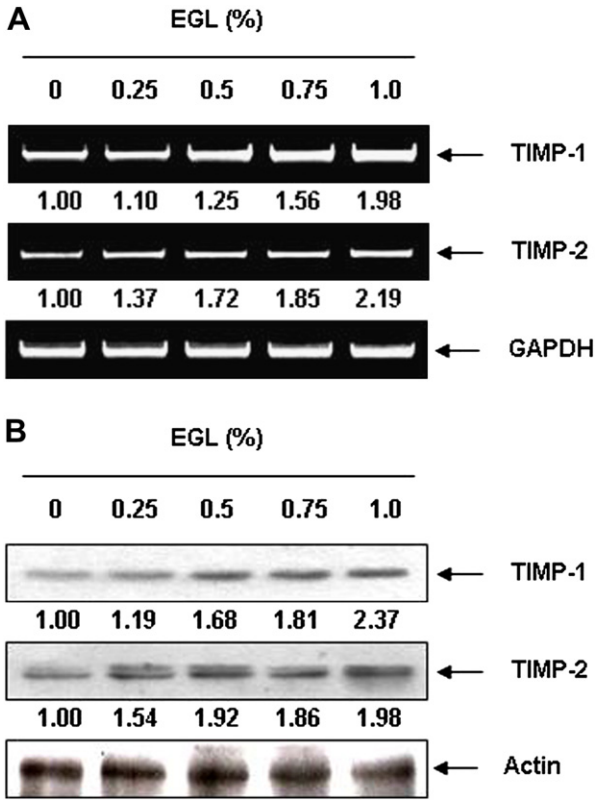

Figure 4 Induction of tissue inhibitors of metalloproteinases (TIMPs) expression by ethanol extracts of *G lucidum* (EGL) in AGS cells. (A) Cells were treated with the indicated concentrations of EGL for 48 hours. Total RNAs were isolated and reverse transcribed. Resulting cDNAs were then subjected to polymerase chain reaction (PCR) and the reaction products were subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. GAPDH was used as an internal control. (B) Cells grown under the same conditions as (A) were sampled, lysed, and 50 μg of proteins were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Western blotting was then performed with anti-TIMP-1 and anti-TIMP-2 antibodies and an enhanced chemiluminescence detection system. Actin was used as an internal control. The relative increase ratios of expression in the results of the RT-PCR and western blotting were presented at the bottom of each of the results as relative values of the GAPDH and actin expression.

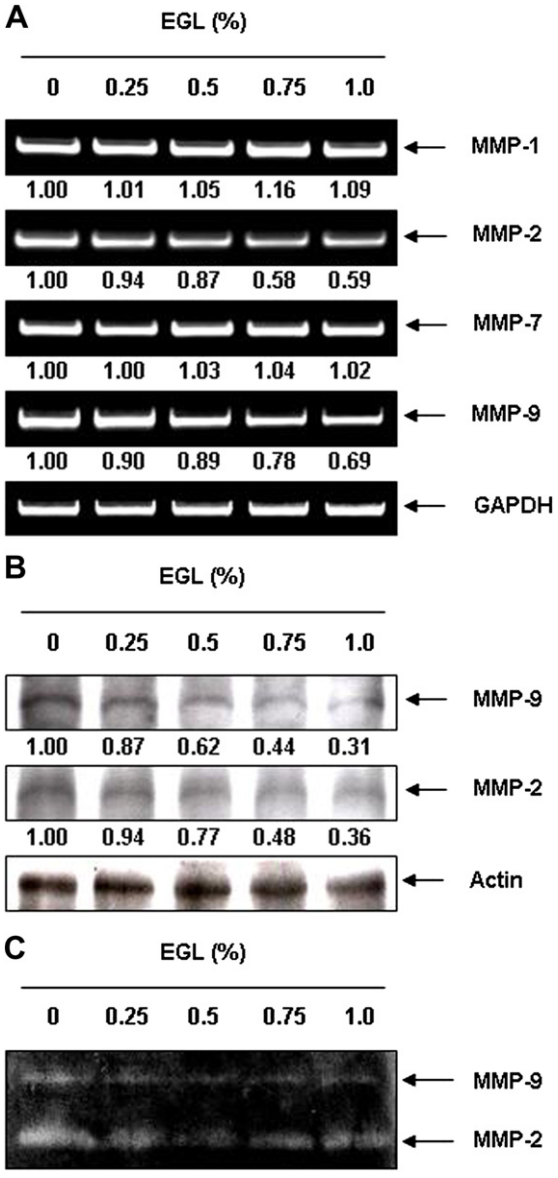

Figure 5 Inhibition of matrix metalloproteinases (MMPs) expression and activity by ethanol extracts of *G lucidum* (EGL) in AGS cells. (A) Cells were treated with the indicated concentrations of EGL for 48 hours. Total RNAs were isolated and reverse transcribed. Resulting cDNAs were subjected to polymerase chain reaction (PCR), and the reaction products were electrophoresized on a 1% agarose gel and then visualized by ethidium bromide staining. GAPDH was used as an internal control. (B) Cells grown under the same conditions as (A) were lysed and 50 μg of proteins were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Western blotting was then performed with anti-MMP-2 and anti-MMP-9 antibodies and an enhanced chemiluminescence detection system. Actin was used as an internal control. (C) After incubation with EGL under the same conditions as those of (A), the medium was collected, and the activities of MMP-2 and MMP-9 were measured by zymography as described in Section 2. The relative ratios of expression in the results of the RT-PCR and western blotting were presented at the bottom of each of the results as relative values of the GAPDH and actin expression.

transcription factor [34,35], were concentration-dependently increased.

4. Discussion

Metastasis is a major barrier to the treatment of cancer and a single event that results in the death of most patients with cancer. Metastasis is the sequential multistep process of the spread of cancer cells and the formation of new tumors in tissues and organs beyond where the original tumor was found. The process ultimately leads to the outgrowth of cancer from one organ to another. Because cancer cell invasion and migration are crucial steps during

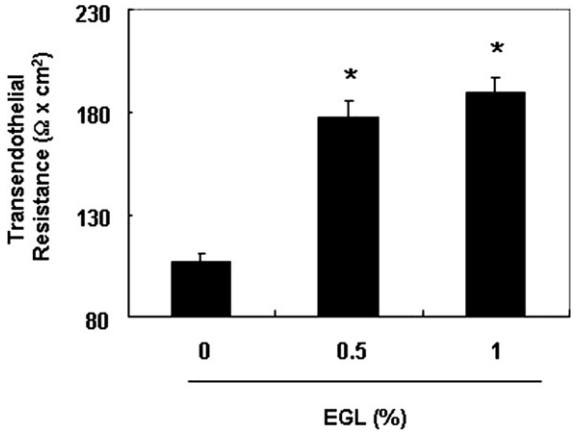

Figure 6 Increase of transepithelial electrical resistance (TER) values by ethanol extracts of *G lucidum* (EGL) in AGS cells. Cells were treated with the indicated concentrations of EGL for 48 hours, and TER values were measured using an EVOM Epithelial Tissue Voltohmmeter as described in Section 2. Results are shown as the mean ± SD of three independent experiments. Significance was determined using a Student *t* test. * $p < 0.05$ versus untreated control.

metastasis, their inhibition is important for anticancer drugs. The aim of this study was to investigate whether *G lucidum* extract has potent anti-invasion and antimetastasis activities in human gastric adenocarcinoma AGS cells. We found that EGL markedly inhibited cell motility and invasive activity through tightening TJs and decreasing MMP activity.

Endopeptidase MMPs are important proteolytic enzymes during organ development and tissue regeneration; however, they also play important roles in cancer invasion and metastasis. Above all, MMP-2 and MMP-9 play important roles in tumor invasion and angiogenesis; therefore, tumor metastasis can be inhibited by blocking MMP synthesis and activity [6,7]. Many researchers have reported that the antimetastatic actions of natural products, including phytochemical agents, were associated with a reduction in MMP-2 and MMP-9 activity [36—39]. MMP activity is tightly controlled by transcriptional activation, a complex proteolytic activation cascade, and an endogenous system of TIMPs. TIMPs inhibit the MMPs by forming 1:1 stoichiometric complexes to regulate matrix turnover [9,35].

Because treatment with less than 1.0% of EGL, which was not cytotoxic as determined by MTT assay, markedly inhibited the cell motility and invasive activity in AGS cells, we investigated whether the inhibitory effects of EGL were associated with the expression modulation of TIMPs and MMPs or changes in their activities. Our results indicated that EGL induced a marked inhibition of MMP-2 and MMP-9 mRNA and protein levels, as well as their enzymatic activities, in a concentration-dependent manner. By contrast, the results of RT-PCR showed that EGL induced a concentration-dependent increase of TIMP-1, as well as TIMP-2 mRNA levels, which was connected with concurrent upregulation of their protein levels, as determined by western blotting. The present data demonstrated that the EGL-induced inhibition of cell motility and invasion is related to downregulation of MMP-2 and MMP-9 activities through the elevation of TIMP expression. Therefore, our results suggest that EGL may increase the TIMPs/MMPs ratio, a key factor in

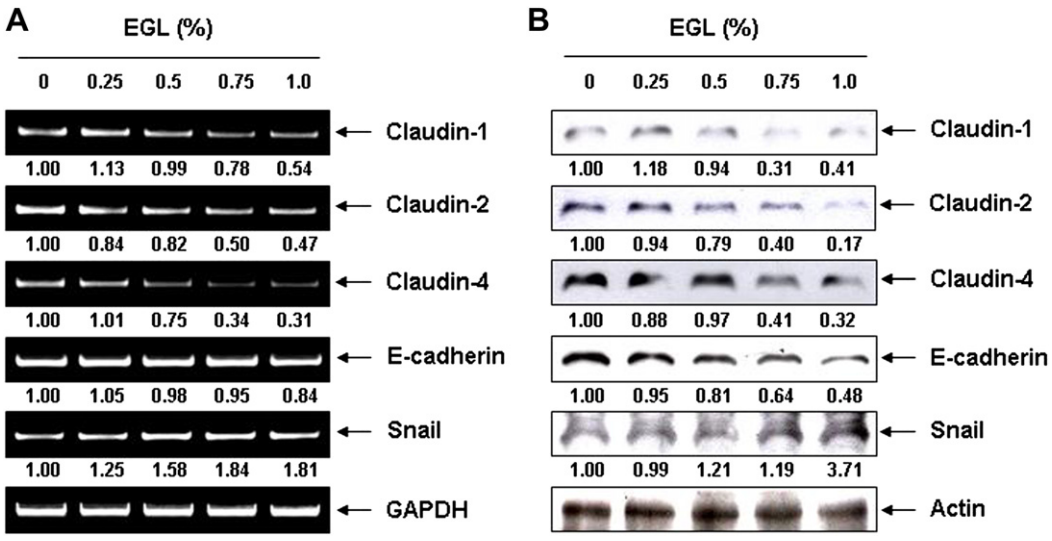

Figure 7 Effects of ethanol extracts of *G lucidum* (EGL) on the expression of claudins, E-cadherin, and snail in Hep3B cells. (A) Cells were treated with the indicated concentrations of EGL for 48 hours. Total RNAs were isolated and reverse transcribed using the indicated primers. Resulting cDNAs were subjected to polymerase chain reaction (PCR), and the reaction products were electrophoresized on a 1% agarose gel and then visualized by ethidium bromide staining. GAPDH was used as an internal control. (B) Cells grown under the same conditions were lysed and equal amounts (50 μg) of cell lysate were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Western blotting was then performed with the indicated antibodies and an enhanced chemiluminescence detection system. Actin was used as an internal control. The relative ratios of expression in the results of the RT-PCR and western blotting were presented at the bottom of each of the results as relative values of the GAPDH and actin expression.

regulating the antimetastatic process, which may then subsequently block the degradation of ECM and lead to the inhibition of cell invasion.

Recently, increasing evidence has indicated that the suppression of the malignant phenotype of cells in tumorigenesis is an additional and important function of the TJs [3]. Moreover, it is becoming increasingly clear that the development of human cancer is frequently associated with the failure of epithelial cells to form TJs and to establish correct apicobasal polarity [40]. This suggests that changes in permeability properties and the loss of cell polarity are hallmarks of epithelial cell tumorigenesis. Thus, it has been demonstrated that TJs, the structures crucial for the maintenance of these functions in epithelial cells, are modulated in a number of epithelial cancers, including gastric cancer [10,11]. These observations indicate that the disruption of the TJ and the dysregulation of its composite proteins play crucial roles in cancer progression, invasion, and metastasis.

Indeed, early studies have demonstrated that the structures of TJ are altered in many epithelial cancers. For example, Soler et al. [10] first demonstrated that the TER of colon carcinoma tissue was significantly lower than that of normal colon tissues but showed higher transepithelial paracellular permeability, which confirmed the loss of the TJs. Other studies have shown that many anticancer agents are inhibitory to motility and invasiveness and that they act by enhancing transepithelial paracellular permeability [29,38,41,42]. In the present study, our results clearly showed that treatment with EGL increased the TER values of AGS cells in a dose-dependent manner: an effect associated with the inhibition of motility and invasiveness. These results indicate that EGL may prevent or reverse TJ leakiness.

Because TJ leakiness is associated with cancer progression and invasion, TJ tightening may have antimetastatic activity [43]. The anti-invasive activity of EGL may be due, in part, to its ability to enhance TJ activity. TJ structure is representative of the conglomerate of molecules that constitute, associate with, or regulate TJs. A number of proteins, as components of TJs, were identified. Among these, 24 members of the claudin family were identified. Claudins are transmembrane proteins with extracellular domains. They form homodimers or heterodimers with other claudins to produce paired strands between cells to regulate paracellular permeability [12].

Previous evidence has indicated that the disruption of TJs, with the concomitant dysregulation of TJ proteins, is an early event in cancer cell invasion and metastasis, and the nature of the dysregulation is highly cancer type-specific. For example, claudin-1 and claudin-7 are downregulated in invasive ductal carcinomas of the breast, as well as in most established breast cancer cell lines [44]. However, claudin-3 and claudin-4 are overexpressed in breast cancers [31,44,45], and other cancers, including gastric [46], ovarian [47], and pancreatic cancer [48]. Conversely, "knockdown" of these two claudins (claudin-3 and claudin-4) inhibited the invasiveness of cancer cells [49].

We also recently showed that claudin-1 plays a causal role in the acquisition of invasive capacity in human liver cells, which was associated with increased expression of MMP-2. However, small interfering RNA targeting of claudin-1 in invasive hepatocellular carcinoma cells completely inhibited cell invasion [50]. These observations indicate that claudins are dysregulated in many types of cancers and may prove to be useful as biomarkers for the detection and diagnosis of certain cancers.

Interestingly, Miyamori et al. [51] reported that claudin promotes the activation of proMMP-2 mediated by membrane-type MMPs. In another study, Agarwal et al. [49] reported that the overexpression of claudin-3 and claudin-4 proteins is associated with increased MMP-2 activity. In addition, Ip et al. [52] showed that the downregulation of claudin-10 reduced MMP activity in human hepatocarcinoma cells. These reports imply a close relationship between MMP activity, the overexpression of claudin, and the metastasis of cancer cells. Therefore, we investigated the effects of EGL on various levels of claudin family members, and the data showed that EGL significantly inhibited the expression of claudin proteins, such as claudin-1, claudin-2 and claudin-4 at both the transcriptional and translation levels. The data suggest that the anti-invasive activity of EGL is associated with the tightening of TJs through the downregulation of claudin family members.

In addition to TJs, cell—cell adhesion in epithelial cell sheets is also maintained through AJs. Thus, we further examined the effect of EGL on the expression levels of a major AJ component, E-cadherin, which is altered or dysregulated in various carcinomas and known to regulate partially the TJ formation [53]. We also investigated the question of whether EGL induced alteration of the levels of snail, an epithelial to mesenchymal regulator, which can lead to decreased cell—cell adhesion and increased cell invasiveness in conjunction with the loss of E-cadherin expression in many types of human cancers [34,35]. As indicated in the results, the levels of E-cadherin were gradually downregulated by EGL treatment in

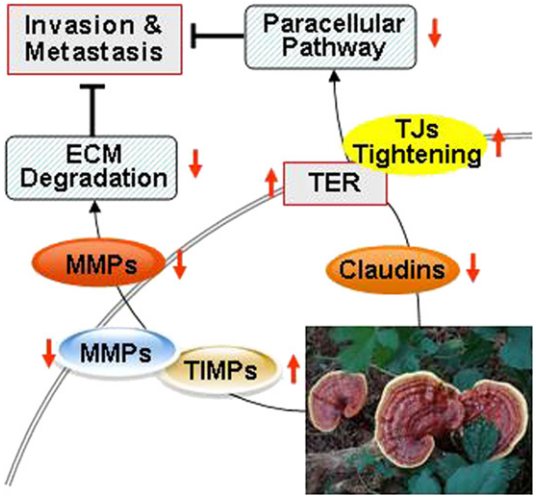

Figure 8 Putative antimetastatic mechanism induced by ethanol extracts of *G lucidum* (EGL) in AGS cells. EGL inhibits cell motility and invasion of AGS cells via tightening of tight junctions and inhibition of the activity of matrix metalloproteinases.

a concentration-dependent fashion. However, those of snail were dose-dependently induced in EGL-treated AGS cells. Although we need to validate this study, we tentatively suggest that EGL, through effects on expression of these proteins, may mediate antimetastasis and anti-invasiveness in AGS cells.

Based on our present data, we suggest a model, as shown in Fig. 8, to explain the EGL-inhibited cell migration and invasion mechanisms in AGS cells. The present results revealed that EGL has an antimetastatic property, which is accompanied by the tightening of TJs and the repression of MMPs activities while concurrently inhibiting claudin expression, as well as inducing TIMPs expression. Our findings point to a novel anticancer mechanism by which *G lucidum* extracts, and the results also indicate that *G lucidum* extracts may be a promising new dietary source for decreasing the risk of developing gastric cancer.

Acknowledgments

This work was supported by a Korea Pharmcopuncture Institute grant and a grant from the Blue-Bio Industry RIC at Dong-Eui University as a RIC (08-06-07) program of KIAT under Ministry of Knowledge Economy, Republic of Korea.

References

1. Hart IR, Goode NT, Wilson RE. Molecular aspects of the metastatic cascade. *Biochem Biophys Acta*. 1989;989:65—84.
2. Jiang WG, Puntis MCA, Hallet MB. The molecular and cellular basis of cancer invasion and metastasis and its implications for treatment. *Br J Surg*. 1994;81:1576—1590.
3. Martin TA, Jiang WG. Loss of tight junction barrier function and its role in cancer metastasis. *Biochim Biophys Acta*. 2009;1788: 872—891.
4. Duffy MI, Maguire TM, Hill A, McDermott E, O'Higgins N. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Res*. 2000;2:252—257.
5. Vihinen P, Ala-aho R, Kähäri VM. Matrix metalloproteinases as therapeutic targets in cancer. *Curr Cancer Drug Targets*. 2005; 5:203—220.
6. Matrisian LM. The matrix-degrading metalloproteinases. *Bioessays*. 1992;14:455—463.
7. Mook OR, Frederiks WM, Van Noorden CJ. The role of gelatinases in colorectal cancer progression and metastasis. *Biochim Biophys Acta*. 2004;1705:69—89.
8. Usami Y, Satake S, Nakayama F, Matsumoto M, Ohnuma K, Komori T, et al. Snail-associated epithelial-mesenchymal transition promotes oesophageal squamous cell carcinoma motility and progression. *J Pathol*. 2008;215:330—339.
9. Lambert E, Dasse E, Haye B, Petitfrere E. TIMPs as multifacial proteins. *Crit Rev Oncol Hematol*. 2004;49:187—198.
10. Soler AP, Miller RD, Laughlin KV, Carp NZ, Klurfeld DM, Mullin JM. Increased tight junctional permeability is associated with the development of colon cancer. *Carcinogenesis*. 1999; 20:1425—1431.
11. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol*. 2004;286: C1213—C1228.
12. Morin PJ. Claudin proteins in human cancer: promising new targets for diagnosis and therapy. *Cancer Res*. 2005;65: 9603—9606.
13. Kominsky SL, Argani P, Korz D, Evron E, Raman V, Garrett E, et al. Loss of the tight junction protein Claudin-7 correlates with histological grade in both ductal carcinoma *in situ* and invasive ductal carcinoma of the breast. *Oncogene*. 2003;22: 2021—2033.
14. Mahajna J, Dotan N, Zaidman BZ, Petrova RD, Wasser SP. Pharmacological values of medicinal mushrooms for prostate cancer therapy: the case of *Ganoderma lucidum*. *Nutr Cancer*. 2009;61:6—26.
15. Boh B, Berovic M, Zhang J, Zhi-Bin L. *Ganoderma lucidum* and its pharmaceutically active compounds. *Biotechnol Annu Rev*. 2007;13:265—301.
16. Paterson RR. *Ganoderma* - a therapeutic fungal biofactory. *Phytochemistry*. 2006;67:1985—2001.
17. Yuen JW, Gohel MD. Anticancer effects of *Ganoderma lucidum*: a review of scientific evidence. *Nutr Cancer*. 2005;53:11—17.
18. Chen JG, Yang CP, Cammer M, Horwitz SB. Gene expression and mitotic exit induced by microtubule-stabilizing drugs. *Cancer Res*. 2003;63:7891—7899.
19. Chien CM, Cheng JL, Chang WT, Tien MH, Tsao CM, Chang YH, et al. Polysaccharides of *Ganoderma lucidum* alter cell immunophenotypic expression and enhance CD56 + NK-cell cytotoxicity in cord blood. *Bioorg Med Chem*. 2004;12: 5603—5609.
20. Lin ZB. Cellular and molecular mechanisms of immunomodulation by *Ganoderma lucidum*. *J Pharmacol Sci*. 2005; 99:144—153.
21. Hu H, Ahn NS, Yang X, Lee YS, Kang KS. *Ganoderma lucidum* extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell. *Int J Cancer*. 2002;102:250—253.
22. Sliva D, Labarrere C, Slivova V, Sedlak M, Lloyd FP Jr, Ho NW. *Ganoderma lucidum* suppresses motility of highly invasive breast and prostate cancer cells. *Biochem Biophys Res Commun*. 2002;298:603—612.
23. Lin SB, Li CH, Lee SS, Kan LS. Triterpene-enriched extracts from *Ganoderma lucidum* inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. *Life Sci*. 2003; 72:2381—2390.
24. Jiang JH, Slivova V, Valachovicova T, Harvey K, Sliva D. *Ganoderma lucidum* inhibits proliferation and induces apoptosis in human prostate cancer cells PC-3. *Int J Oncol*. 2004;24:1093—1099.
25. Hong KJ, Dunn DM, Shen CL, Pence BC. Effects of *Ganoderma lucidum* on apoptotic and anti-inflammatory function in HT-29 human colonic carcinoma cells. *Phytother Res*. 2004;18: 768—770.
26. Stanley G, Harvey K, Slivova V, Jiang J, Sliva D. *Ganoderma lucidum* suppresses angiogenesis through the inhibition of secretion of VEGF and TGF-β1 from prostate cancer cells. *Biochem Biophys Res Commun*. 2005;330:46—52.
27. Müller CI, Kumagai T, O'Kelly J, Seeram NP, Heber D, Koeffler HP. *Ganoderma lucidum* causes apoptosis in leukemia, lymphoma and multiple myeloma cells. *Leuk Res*. 2006;30: 841—848.
28. Jang KJ, Han MH, Lee BH, Kim BW, Kim CH, Yoon HM, et al. Induction of apoptosis by ethanol extracts of *Ganoderma lucidum* in human gastric carcinoma cells. *J Acupunct Meridian Stud*. 2010;3:24—31.
29. Kim SO, Choi BT, Choi IW, Cheong J, Kim GY, Kwon TK, et al. Anti-invasive activity of histone deacetylase inhibitors via the induction of Egr-1 and the modulation of tight junction-related proteins in human hepatocarcinoma cells. *BMB Rep*. 2009;42: 655—660.
30. Lee YR, Noh EM, Jeong EY, Yun SK, Jeong YJ, Kim JH, et al. Cordycepin inhibits UVB-induced matrix metalloproteinase expression by suppressing the NF-κB pathway in human dermal fibroblasts. *Exp Mol Med*. 2009;41:548—554.
31. Kominsky SL. Claudins: emerging targets for cancer therapy. *Expert Rev Mol Med*. 2006;8:1—11.

32. Nelson WJ, Nusse R. Convergence of Wnt, β-catenin, and cadherin pathways. *Science*. 2004;303:1483—1487.
33. McLachlan RW, Yap AS. Not so simple: the complexity of phosphotyrosine signaling at cadherin adhesive contacts. *J Mol Med*. 2007;85:545—554.
34. Yokoyama K, Kamata N, Hayashi E, Hoteiya T, Ueda N, Fujimoto R, et al. Reverse correlation of E-cadherin and snail expression in oral squamous cell carcinoma cells *in vitro*. *Oral Oncol*. 2001;37:65—71.
35. Uzui H, Harpf A, Liu M, Doherty TM, Shukla A, Chai N. Increased expression of membrane type 3-matrix metalloproteinase in human atherosclerotic plaque: role of activated macrophages and inflammatory cytokines. *Circulation*. 2002;106:3024—3030.
36. Choi YH, Choi WY, Hong SH, Kim SO, Kim GY, Lee WH, et al. Anti-invasive activity of sanguinarine through modulation of tight junctions and matrix metalloproteinase activities in MDA-MB-231 human breast carcinoma cells. *Chem Biol Interact*. 2009;179:185—191.
37. Moon DO, Choi YH, Moon SK, Kim WJ, Kim GY. Butein suppresses the expression of nuclear factor-kappa B-mediated matrix metalloproteinase-9 and vascular endothelial growth factor in prostate cancer cells. *Toxicol In Vitro*. 2010;24: 1927—1934.
38. Shin DY, Kim GY, Kim JI, Yoon MK, Kwon TK, Lee SJ, et al. Anti-invasive activity of diallyl disulfide through tightening of tight junctions and inhibition of matrix metalloproteinase activities in LNCaP prostate cancer cells. *Toxicol In Vitro*. 2010;24: 1569—1576.
39. Shin DY, Ryu CH, Lee WS, Kim DC, Kim SH, Hah YS, et al. Induction of apoptosis and inhibition of invasion in human hepatoma cells by anthocyanins from meoru. *Ann N Y Acad Sci*. 2009;1171:137—148.
40. Latorre IJ, Roh MH, Frese KK, Weiss RS, Margolis B, Javier RT. Viral oncoprotein-induced mislocalization of select PDZ proteins disrupts tight junctions and causes polarity defects in epithelial cells. *J Cell Sci*. 2005;118:4283—4293.
41. Verghese GM, Gutknecht MF, Caughey GH. Prostasin regulates epithelial monolayer function: cell-specific Gpld1-mediated secretion and functional role for GPI anchor. *Am J Physiol Cell Physiol*. 2006;291:C1258—C1270.
42. Kim SO, Kwon JI, Jeong YK, Kim GY, Kim ND, Choi YH. Induction of Egr-1 is associated with anti-metastatic and anti-invasive ability of β-lapachone in human hepatocarcinoma cells. *Biosci Biotechnol Biochem*. 2007;71:2169—2176.
43. Mullin JM, Agostino N, Rendon-Huerta E, Thornton JJ. Keynote review: epithelial and endothelial barriers in human disease. *Drug Discov Today*. 2005;10:395—408.
44. Kominsky SL, Vali M, Korz D, Gabig TG, Weitzman SA, Argani P, et al. Clostridium perfringens enterotoxin elicits rapid and specific cytolysis of breast carcinoma cells mediated through tight junction proteins Claudin 3 and 4. *Am J Pathol*. 2004;164: 1627—1633.
45. Tokés AM, Kulka J, Paku S, Szik A, Páska C, Novák PK, et al. Claudin-1, -3 and -4 proteins and mRNA expression in benign and malignant breast lesions: a research study. *Breast Cancer Res*. 2005;7:R296—R305.
46. Cunningham SC, Kamangar F, Kim MP, Hammoud S, Haque R, Iacobuzio-Donahue CA, et al. Claudin-4, mitogen activated protein kinase kinase 4, and stratifin are markers of gastric adenocarcinoma precursor lesions. *Cancer Epidemiol Biomarkers Prev*. 2004;15:281—287.
47. Rangel LB, Agarwal R, D'Souza T, Pizer ES, Alò PL, Lancaster WD, et al. Tight junction proteins claudin-3 and claudin-4 are frequently overexpressed in ovarian cancer but not in ovarian cystadenomas. *Clin Cancer Res*. 2003;9: 2567—2575.
48. Michl P, Barth C, Buchholz M, Lerch MM, Rolke M, Holzmann KH, et al. Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. *Cancer Res*. 2003;63: 6265—6271.
49. Agarwal R, D'Souza T, Morin PJ. Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. *Cancer Res*. 2005;65:7378—7385.
50. Yoon CH, Kim MJ, Park MJ, Park IC, Hwang SG, An S, et al. Claudin-1 acts through c-Abl-protein kinase Cdelta (PKCδ) signaling and has a causal role in the acquisition of invasive capacity in human liver cells. *J Biol Chem*. 2010;285: 226—233.
51. Miyamori H, Takino T, Kobayashi Y, Tokai H, Itoh Y, Seiki M, et al. Claudin promotes activation of pro-matrix metalloproteinase-2 mediated by membrane-type matrix metalloproteinases. *J Biol Chem*. 2001;276:28204—28211.
52. Ip YC, Cheung ST, Lee YT, Ho JC, Fan ST. Inhibition of hepatocellular carcinoma invasion by suppression of Claudin-10 in HLE cells. *Mol Cancer Ther*. 2007;6:858—2867.
53. Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. *Annu Rev Physiol*. 2006;68:403—429.